

ORGAN EXTRACTS

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A Dissertation Submitted in Partial Fulfillment of
the Requirements for the Degree of Doctor
of Public Health in the University
of Michigan.

Reprint from

THE JOURNAL OF
LABORATORY AND CLINICAL MEDICINE
St. Louis

Vol. IV, No. 9, June, 1919



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HISTORICAL REVIEW

In 1888 Brieger isolated a specific, active, toxic substance from the amputated arm of a victim of tetanus. In the year following Brieger and Frankel noted the toxalbumin in broth cultures of *B. Tetani*. Kitasato in 1891 injected the blood of tetanus infected laboratory animals into other animals, and Nissen did the same with blood from patients dying from a tetanus infection, whereby both were able to observe its toxic action. Brieger's pupil, Immerwahr, went a step farther than his teacher and isolated a toxalbumin from the organs of infected laboratory animals which he assumed to be identical with the product from the bouillon cultures. He likewise isolated the same substance from an amputated human leg. Stern in 1892 demonstrated the toxic activity of the blood of tetanus patients fifty-four hours before their death. Then a large number of workers began to investigate the blood stream of human beings under all sorts of pathologic conditions as well as in health. Rabbits were used as recipients and the M.L.D. of normal human blood varied among some fifteen workers between 8 and 27 c.c. per kilogram of rabbit. The blood of sheep, swine, the ox and horse were likewise injected and their toxicities determined.†

Uhlenuth¹ in 1897 reviewed these divergent observations, and after amplifying laboratory trials to include a number of new diseases and pathologic conditions, concluded that positive animal reactions to the injection of blood could not be relied upon as differentiating between a normal and a pathologic con-

*From the Hygienic Laboratory, University of Michigan.

†Quoted from Uhlenuth: *Ztschr. f. Hyg.*, 1897, xxvi, 384-397.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Public Health.

dition of the donor's blood. He made his injections subcutaneously on guinea pigs and found that all blood, in greater or less quantity, caused infiltrations at the site of the injection when in small doses, with necroses followed by death when the doses were relatively large. Normal horse serum was the least toxic and almost without effect up to a dose of 20 c.c. when an easily resorbed infiltration was evident. Obduction of dead animals disclosed nothing of any importance except in the animals dying from an injection from a scarlet fever patient, when a cloudy swelling of the kidneys was noted, accompanied by incipient fatty changes.

In 1898 Brieger and Uhlenuth made inquiry into the nature of the toxic agent that seemed to be present in all bloods, and decided that it had nothing to do with the fibrin ferment of Schmidt and Pekelharing, or with any other ferment that they knew anything about, and that it did belong to the toxalbumins, since it was readily destroyed by alcohol, and was refractory to dialysis and filtration through porcelain. They next tried the effect of placing a bit of homologous tissue under the skin, and found that similar necroses developed, usually ending in the death of the animal. A physiologic salt suspension of the tissue was found to be equally effective. Greatly to their surprise, when they extended their work to the heterologous tissues, they found that the tissues of the horse, to whose serum the animals failed to react, were very powerful in their necrotic effects. On the other hand no differences could be detected in the effects of normal human or pathologic tissues of any sort. Plunging into this work with considerable energy they soon found that a more thorough preparation of the tissues before placing them under the skin permitted them to be absorbed without appreciable effects, so they dropped the work at that point.²

In 1909 Schenk³ expressed a liquid from placental tissue which had a toxic action upon intravenous injection into rabbits. He was seeking some light on eclampsia. He found that a short incubation with a small quantity of serum from varying sources sufficed to completely detoxify the expressed liquid. Inactivation of the sera did not affect their ability to render harmless the extracts. He was able to secure a short immunity to as high as three lethal doses by the usual process of repeated sublethal injections.

Kraus, Volk, and Lowenstein⁴ in 1911, working in the field of tuberculin, thought that animals infected with tuberculosis showed a marked susceptibility to a liquid which they had expressed from the organs of tuberculous laboratory animals. However, further researches showed that there was no specific susceptibility on the part of the infected animals, and also that liquids expressed from the organs of healthy animals served to produce the same necroses and deaths.

In Dold's⁵ initial paper he reviewed much of the work that has been mentioned and confirmed that of Volk and Lowenstein in particular. He worked with physiologic salt extracts and pointed out that the toxicity of the extracts varied directly with the quantity of tissue employed or inversely to the amount of salt solution used. He found that the intraperitoneal or subcutaneous dose

was from three to four times that required by intravenous injection for the same result, and that the homologous species was relatively more susceptible to the different tissue extracts than heterologous species. He further noted that extracts lost their toxicity not only by standing at room temperature for a few days, again confirming Volk and Lowenstein, but also by heating to 60° C. for not less than thirty minutes. Such heating caused the formation of a precipitate, the decrease in toxicity being parallel with the mass of the precipitate that was thrown down. Filtration through a Berkefeld also rendered Dold's extracts nontoxic; but Aronson⁶ had already noted this point in 1909 in connection with an extract of beef kidneys that he had prepared for the treatment of kidney disorders. Dold also used serum for the detoxification of extracts without acknowledgment of Schenk's earlier work which provoked a polemic concerning the priority of the work on organ extracts. Schenk⁷ claimed that his "Pressäfte" was a general principle and that Dold had done nothing new when he substituted extraction for pressure or extended the work to other tissues. However, Dold did determine more closely the quantity of serum that would detoxify an extract and found ³¹ that so small a quantity as .5 c.c. in contact with a lethal dose of the extract for fifteen minutes completely neutralized its effects. In continuing his work Dold⁸ noted that all sera were not equal in their power for detoxification, but speaking broadly, the homologous serum with the tissue was most effective. No definite law could be formulated because all sera in some proportion would minimize the toxic effect of all extracts. Filtering the sera through porcelain completely eliminated their power to remove the active agent from the extracts. Dold believed the process of detoxification to partake of the nature of a ferment inhibition, pointing in support of this view to the work of Hedin⁹ who had observed that the addition of egg or serum albumin to a casein-trypsin mixture hampered digestion, and that the inhibition was more complete when the order of combination was trypsin-albumin-casein. Dold further showed that complement did not enter into the neutralization process, for by appropriate titration, complement was shown to be undiminished in a serum that had been used for detoxification as compared to an unused portion of the same serum.

Incubation with kaolin removed the toxicity of the extracts, and Izar¹⁴ later showed that brain emulsion, talcum, animal charcoal, and rice starch would in particular amounts for each substance render the extracts harmless. When Dold attempted to immunize rabbits by successive sublethal doses the animals rapidly became cachectic and died at varying intervals. The best he could do was to obtain a very feeble resistance to one lethal dose in some animals. This is in marked contrast to Schenk's earlier claim of immunity to three lethal doses of his expressed liquid.

Inquiring into the source of the toxic substance that seemed to be ubiquitous for animal tissues, Dold¹⁰ assumed that it had its origin in either the body cells or in the lymph. He could obtain but weakly toxic extracts from washed leucocytes or erythrocytes, and injections of relatively large quantities of either the fresh cells or the cell residues after extraction, failed to produce

an effect that was at all comparable to that of the tissue extracts. The animals died, but the preliminary symptoms were not the same and the deaths were delayed a considerable time as compared to the three to eight minute deaths of the animals receiving the tissue extracts. Dold dissected ox and rabbit eyes into their components and made extracts of the separate parts. He found the toxicity of these extracts to be parallel with their cell content. The order of toxicity in a descending scale being, uvea and retina, sclera, cornea, lens, and vitreous humor, the latter two being entirely without effect. He explains the apparent discrepancy between these findings and his assertions concerning the red and white cells by calling attention to the more strict metabolic activities of the tissue cells as compared to the functions of the blood cellular elements. Cutting a rabbit's lung into three pieces, he allowed it to remain in physiologic salt solution for two hours. After removal of the lung the solution was centrifuged, and upon injection proved to be as toxic as if the tissue had been rubbed up as was his usual custom. His conclusion as to the source of the poison is best expressed by his statement: "An extractable poison is in animal tissue in a quantity proportional to their metabolic activities and lymph content."

Animals receiving a lethal dose of organ extracts present the characteristic features of the anaphylactic syndrome. However, obduction shows clots in the heart, accompanied by abdominal hyperemia and thrombi in the lungs. Dold believed the thrombi to be the cause of death. In order to test the point he added Jacoby's hirudin to a lethal dose of the extract or else gave the animal an injection of hirudin just preceding the injection of the extract. The clots were not found in the lungs in spite of the fact that the animals died. This indicated that the supposed thrombic enzyme did not play the part assumed by Dold. A further use of a more freshly prepared extract of leeches not only saved the animals, but presumably prevented the formation of the thrombi. One can therefore form his own conclusions as to the formation and influence of the thrombi, since Dold did not attempt to further clear up the point. He found that ten per cent peptone administered in varying quantities just before the injection of the extracts saved, from a considerable series of experimental animals, an average of 75 per cent.

Bauer and Wüstoff¹¹ considered organ extracts to be equivalent to anaphylatoxin as prepared *in vitro*. Dold proposed the same notion and even thought that tissue from anaphylactic animals yielded a more typical anaphylatoxin.³²

Gräfenberg and Thies¹² made a physiologic salt extract of testicles and found it to be quite toxic. They were interested in the specificity of tissue reactions as compared to species reactions to such extracts. They found that males of the same species were slightly more susceptible than any normal animals; females of the same species were highly resistant. Young animals of the same species showed no sexual differences in their reactions to the extracts. The most susceptible animals to the homologous testicular extracts were castrated males and pregnant females. Sensitized animals, which they were able to secure by careful manipulation, reacted promptly to an injection of any

extract. They could find no grounds for a species specificity, but believed their work to point to a possibility of tissue specificity to testicular extracts. Izar and Faguoli¹³ made a methyl alcohol extract of bovine and dog testicles and injected into rabbits and pigs a watery emulsion of the extracted lipoids. They used a long series of dilutions and observed but slight evidences of toxicity. Heating such emulsions for one-half to one hour at a temperature of 50° C. raised their toxicity very markedly. Carrying the heating on up to a temperature of 100° C. for ten minutes still caused an increase in toxicity, but if heated longer at this higher temperature, the toxicity fell below that for the unheated extracts. A previous injection of .8 to 2.5 c.c. saturated sodium chloride containing one per cent of calcium chloride protected rabbits from death in most cases and always prolonged it. Titration of complement before and after injection of their watery emulsions showed a marked decrease following the injections, and the decrease was in general parallel to the severity of the reaction to the emulsion. They confirmed Dold's failure to secure immunity.

Izar and Ascoli³⁰ and later Izar¹⁴ alone, worked with lung extracts of pigeons, sparrows, and rabbits. They noted the temperature reactions and found them to be typical for the parenteral injection of proteins. They extended the attempt to protect the recipient animals by a previous or simultaneous injection of inorganic salts, using salts of barium, strontium, magnesium, potassium, ammonium, and cadmium. Such protection as they were able to observe they ascribed to the cations involved. The various salts were not uniform in their effects and they found calcium chloride the most effective, because it lowered the toxicity more and therefore kept the temperature curve more nearly normal. They found serum from well fed animals to be most potent in detoxification; although serum from starved animals was not without considerable effect in lowering toxicities and therefore assisting in the maintenance of a more nearly normal temperature curve. Later work by Izar and Patane¹⁵ throws interesting light on Dold's conclusions concerning the source of the poison. They made the usual extract of rabbit's lung and proved its toxicity. The tissue residue from the original extraction was then washed in several liters of physiologic salt solution in successive portions, all the washings being discarded. Then the original technic was repeated on the tissue and the extract thus made proved to be nearly as toxic as the original. Again, the lungs of a freshly bled rabbit were removed and an extract made from one immediately. The other was perfused with salt solution until it was white and bloodless, and then extracted. When both extracts were tested for their toxicity, that from the perfused lung proved to be the more toxic. They found it impossible to secure a toxic extract from the dried and powdered lung with methyl alcohol, although Fischer¹⁶ could detect no differences in the properties of a physiologic salt extract of fresh and dried tissue. Izar and Patane incubated the physiologic salt extract of lungs at 37° C. and found that the toxic principle was quantitatively bound to the precipitate that was thrown down. Continuing Izar's previous work on the protection afforded by the inorganic salts, they were able to neutralize *in vitro* by the addition of tenth normal cal-

cium chloride. However, if to such a neutralized extract they added an exact chemical equivalent of potassium oxalate the toxicity was restored. Their earlier work had shown that potassium ions were low in protection as compared to the ions of calcium.

Fischer could not confirm Dold's observation that standing a few days at room temperature sufficed for the extracts to lose their potency, as his were effective after standing for eight weeks. He asserted that there were two substances that influenced the clotting of blood; one that hastened, and another that delayed the formation of a clot. Therefore, the obduction findings will depend on which phase of these influences was predominant when the animal died. The quantity of extract was but a minor part in the actual working out of its effects, and we are told that the exact nature of the substances that control the blood clotting is as yet unknown.

Distaso¹⁷ worked with material secured from cadavers, also with fresh tissues of laboratory animals. He shortly abandoned the cadaverous material as he satisfied himself that their toxicity was a result of a bacterial activity after death. He found tissue extracts to be heat labile, inactivated by serum, and rendered harmless by filtering through porcelain, thereby confirming Dold's work on these points. He also reported that precipitation with alcohol destroyed the toxic agent; that treatment with fibrin or gelatine markedly lowered their effects, and that dialysis completely inactivated his extracts through the loss of salts. Before abandoning the cadaverous material as a source of tissue extracts, he worked out some of their properties, and since they were almost parallel for those of a ferment such as diastase, he concluded that the active principle of extracts was a colloidal ferment. Later he made a comparative study of extracts made from the lungs and intestines of a female rabbit and her fetal young. He found that the lungs of both yielded a toxic extract, but the intestines of the mother only did so. He argued that this showed the active principles of lung and intestinal extracts to be dissimilar, and that intestines gave a toxic extract by reason of the processes of extra-uterine life. Dale and Laidlaw¹⁹ isolated B-iminazolyethylamine from colon tissue. Its action is very similar to Distaso's intestinal extracts, but it is heat stable, so can not be the same product. Roger¹⁸ found that bile and pancreatic fluids, injected separately or together were highly toxic for the homologous animal. Further, Roger²⁰ and Falloise²¹ have shown that the poisonous material contained in feces is more marked in the excreta of a breast fed infant than from an adult, and that feces of a normal adult are more powerfully toxic than from a typhoid fever patient. In all cases extracts of fecal material were heat stable.

Ichikawa²² in 1913 studied the blood clot accelerating effects of various extracts *in vitro* and found this property to run parallel with their toxicity. He was unable to confirm Dold's assertions that the extracts were most toxic for the homologous animal and that homologous serum was most effective for detoxification. He found that sodium citrate had an effect analogous to hirudin either by previous or simultaneous injection with the extracts. He evidently considered organ extracts to be equivalent, in their effects at least, to anaphy-

latoxin, for he remarks that the temperature changes produced by injection of Friedberger's anaphylatoxin and of appropriate amounts of organ extracts are identical. Injection of large, but sublethal doses of lung extract, caused marked leucopenia; smaller doses caused an incipient leucopenia which soon reversed itself and ended in leucocytosis. Blood pressure remained unaltered by the injection of sublethal doses. No agglutination or precipitation of homologous red cells by lung extracts could be observed by Ichikawa, although Embleton²³ was able to note an appreciable rise in the hemolytic power of serum from rabbits that had received a sublethal dose of some organ extract. It will be recalled that Dold was able to detoxify his extracts by heating to 60° C. for not less than one-half hour; Ichikawa confirmed this point for rabbit lung extract, but that from the lungs of the guinea pig withstood this temperature without any diminution of toxicity. Aronson,²⁴ in attempting to prepare anaphylatoxin without the use of serum, heated bacterial cells in physiologic salt suspension to a temperature of 65° C. and secured a toxic filtrate by centrifuging out the germ substance. If bacteria are chemically equivalent to the extracts of tissue and one can rule out the possibility of the extraction of an endotoxin, that would be heat stable to the temperature employed, it would then be in order to use Aronson's work in checking up other results with organ extracts. Ichikawa gave support to the dual nature of the extracts as suggested by Fischer, by attempting to immunize rabbits with a heat neutralized extract. The heating eliminated the toxicity, with its accompanying blood clotting changes, but the animals all developed cachexia and died just as if they had been given sublethal doses of the unheated extract.

Dold and Kodama²⁵ in a later work indicate that all their extracts were acid in reaction, and that they were not able to modify their toxicity by either making alkaline or slightly more acid. A precipitate settled out upon stronger acidification which was found to carry the toxic agent with it, thereby confirming Izar and Patane in a similar observation. This precipitate could be kept dry for one month and the toxin recovered by water, ether or acetone. Addition of fresh sera to the dried precipitate, followed by removal and an immediate extraction of the substance by water showed that the sera had destroyed the toxic principle. They also added their confirmation as to the effect of alcohol on organ extracts as announced earlier by Distaso. Treatment of an aqueous extract with magnesium sulphate partially salts out the toxic substance, which could be recovered salt free by dialysis. Seeking more knowledge concerning the chemical identity and nature of organ extracts, Dold found that treatment with ether caused a separation into a watery layer on the bottom, with a precipitate above, and the ether on top of all. When very strong extracts were so treated a part of the poison was found in the precipitate, but if an extract of only moderate toxicity was handled with ether the poison was found to have been destroyed. Similar treatment with acetone always destroyed the poison, regardless of what strength of extract was used. Since these same characteristics are also common to ferments, they were held to be additional proof of the probable ferment nature of the extracts.

Catapano²⁶ and Bittorf²⁷ examined a large number of extracts of animal and human tissue for their mydriatic properties. The adrenals were the most active but all tissues with exception of thyroid, spleen, and nerve yielded an extract of greater or less activity. Bingel and Strauss²⁸ confirmed these workers in all their findings except for the kidney and liver. All were agreed that the extracts, as far as their mydriatic properties were concerned, were stable to heating, and the last mentioned workers claimed serum to be without effect on this characteristic. Dold²⁹ probably had occasion to note the same effects on the eye as these workers, because he does mention a marked inflammatory process that he had observed to come on very rapidly, which slowly subsided.

EXPERIMENTAL

First, a brief resumé of the circumstances that provoked this study will be of some interest. In the course of a series of experiments we had occasion to attempt to determine the fate of Vaughan's poisonous protein cleavage product when administered enterally to cats. The presence of the poison in the blood stream was definitely established by heart punctures following its introduction into the stomach by a tube. By withdrawals of heart blood from a dosed cat at intervals of ten to twelve minutes, followed by immediate transfer of such blood to guinea pigs intravenously, a wave of toxicity could be noted that rose to a peak in about one hour and then gradually faded away. This observation was carefully checked by the injection of normal cat's blood. Following the matter one step farther, it seemed in order to see if any of the poison could be recovered from the tissues of such an experimental animal after it had been repeatedly dosed with the poison. For reasons which need not be given here alcoholic extraction of the tissues, followed by appropriate after-treatment of the extract was the method of procedure. Obtaining an extract that was highly toxic to guinea pigs upon intravenous injection it was interpreted as evidence of poison recovery. However, upon applying the identical technic to the organs and tissues of a normal cat an extract was secured that was equally toxic to the recipient guinea pigs, as the extract from the tissues of a dosed cat. Blocked, for the time at least, by this unexpected result, a study of the toxic properties of organs and tissues was undertaken.

Methods Employed.—It will save repetition if the details of the method of extracting the tissues are given at this time. What we have already referred to as the "extract," and will have frequent occasion to refer to again, was prepared by the following procedure:

Animals were killed by thorough exsanguination. Such tissues as were desired were immediately removed and placed in separate receptacles. They were washed, under the tap, free of all blood, and all fat and extraneous tissues were removed from each organ. The stomach and intestines were opened up and well cleaned. By the use of an ordinary household food chopper the tissues were ground to the consistency of a fine sausage. In this condition the tissues were placed, always in separate vessels, under absolute alcohol. The quantity of alcohol was 2 c.c. for each gram of the moist ground tissue. For this purpose Erlenmeyer flasks were used, stoppered with a plug of cotton.

They stood at room temperature with their contents for periods varying from two days to five weeks, occasionally being shaken. At the conclusion of this extraction period with the alcohol it was recovered. This was accomplished by pouring out the flasks upon a clean unstarched gauze supported in a funnel. This funnel was so arranged that the fluid passed onto a qualitative filter paper supported in another funnel below the one containing the cloth. The tissue was well squeezed out by hand compression in the gauze. The alcoholic filtrate was received in a large evaporating dish. The alcohol was driven off on a steam bath, the evaporation being carried nearly to the point of dryness. The residue left in the dish was then taken up either with physiologic salt solution or with distilled water, usually the latter. Macroscopically nothing seemed to be dissolved out of the residue by this treatment. However, color was imparted to the liquid, and this varied in intensity with the tissue concerned. This liquid was cleared by filtration through paper, and the amount of water or of salt solution added was so manipulated that the final volume of the filtrate represented two grams of the original moist tissue to each c.c. The secondary or final extraction fluid was refractory to filtration. This was especially marked in the extracts from the abdominal viscera. In order to obviate this difficulty recourse was sometimes taken to passing the liquid through a plug of glass wool in order to eliminate the larger part of the material in suspension. This filtrate was centrifuged at eight thousand R.P.M. for ten to fifteen minutes. This substitution of centrifugation for filtration produced no essential change in the characteristics of the extracts other than an increased clarity. Evidently whirling at the high speed available would throw down some particles that would pass a filter paper. This was shown to be so by centrifuging an extract that had originally been filtered. A very slight deposit was observed in the centrifuge tubes, accompanied by the change in clearness. However, no change whatever was detected in the toxicity of an extract after such treatment. This final watery solution of such materials as may have been dissolved out of the residue left upon evaporation of the alcoholic extraction liquid, is what we have designated as the extract. In all cases, except as shall be hereafter indicated, it was prepared as outlined.

Method of Testing Toxicity.—The guinea pig was used as the animal of choice for testing toxicity. This animal reacts promptly to injections of toxic substances, and generally with consistency. However, in tabulations of experimental data that are to follow, some instances will be noted where guinea pigs of approximate weights reacted differently to equal doses of the same extract or of other toxic substance. One must therefore consider that individual variation is still playing some part even in as highly susceptible an animal as the guinea pig. This accords with the experiences of others on the same point.³³ The place of injection was into one of the external jugulars, usually the left, the Latapie animal board being employed as a holder. The vessel mentioned is reached readily and nearly bloodlessly by a properly placed superficial incision.

Since the guinea pigs used as recipients for the extracts displayed the true anaphylactic syndrome it will be in order to briefly outline the interpretation of the phases used in tabulations to describe their responses.

Nil means a failure to react. Included in this group are all animals that show only an accelerated breathing rate by reason of the excitement of handling. Distinct pumping was lacking.

Light shock indicates some peripheral irritation, accompanied by hyperexcitability, slight dyspnea and jerks.

Moderate shock conveys the picture of a very rapid passage of the preliminary stages immediately followed by spasms and marked air hunger. The animal if thrown did not remain prostrate, but was soon on its feet again.

Violent shock and *near death* are differentiated only by the severity of the prostration. In those cases where the shock is apparently of maximum intensity, but the animal after a short period of prostration recovers promptly and fully, the first term is used; but when the prostration is long drawn out, during which time there may be some question as to whether the animal is dead or alive, the latter expression is employed.

The twitching of the nose that is so characteristic of anaphylactic death was used to mark fatal termination. Figures given under the result heading in the tables always indicate the death of the animal and give the elapsed time from the completion of the injection to the last nasal twitch.

Autopsies were invariably performed and are only commented upon when the obduction shows something at variance with the typically distended lungs, beating heart, fluid blood and hyperemia of the abdominal viscera.

Distribution of Extractable Poison in Tissues.—It was thought desirable in the beginning to subject a wide range of tissues to the extraction process, and note the occurrence of the toxic agent. By adhering to the same technic it would be possible to make observations as to the probable quantity of the poison present in the tissues examined. The results of this phase of the problem are conveniently summarized in Table I.

The tissues represented in Table I are sufficiently heterogenous, in both respect to species and organs, to warrant the belief that the extractable poisonous agent of tissue is present in all animal tissues to a greater or lesser extent.

Quantity of Material Extracted from the Tissues.—When the alcoholic extract was evaporated the mass of the residue left in the dish was slight. It formed but a very thin coating on the bottom and sides of the dish. It has already been remarked that the treatment of this residue with water did not dissolve any appreciable quantity of it. It was decided to get some exact information as to the actual quantities of material being extracted by the alcohol from the tissues. We wished further to know how much of this material was dissolved by the water, and to determine the amount of solids held in solution in the extract as injected into the guinea pigs. Accordingly, measured amounts of the alcoholic extract were evaporated to dryness on the steam bath and the weight of the residues determined. The same process was carried out on the extract as injected. From these figures it was but a simple procedure to calculate the total residue from a given amount of tissue and therefore the percentage of material that was being extracted. It is obvious the same information could be obtained concerning the extract as it was injected. It will be noted that the concentration of the final extract is expressed in terms of milligrams per cubic centimeter. Table II gives the data on the point under consideration.

The data in Table II are from the tissues of the cat. They are identical with the tissues of the cat the toxicity of which is given in Table I. It is possible to make a comparative study between the amounts of solids that are being injected and the toxicity of the extract. This will be taken up later.

TABLE I

SHOWING THE DISTRIBUTION AND RELATIVE TOXICITY OF A POISONOUS EXTRACTABLE
SUBSTANCE IN TISSUE

SOURCE		GUINEA PIG		C.C.	RESULT
ANIMAL	TISSUE	NO.	WT.	INJECTED*	
Cat	Stomach	1	175	5	3'
		2	175	3	2'
		3	180	1	Light shock
	Intestine	4	150	5	4'
		5	180	1	Light shock
		6	230	5	Violent shock
	Liver	7	190	3	Moderate shock
		8	160	1	Nil
		9	235	5	Violent shock
	Kidney	10	200	3	Violent shock
		11	215	1	Light shock
		12	160	5	2'
	Spleen	13	200	1	Nil
	Lung	14	245	5	Moderate shock
	Heart	15	190	5	Light shock
	Muscle	16	215	4	1' 30"
		17	205	2	Near death
		18	250	1	Nil
	Brain	19	190	5	2'
		20	200	2	Nil
Rabbit	Muscle	21	155	3	2'
		22	180	1	Nil
		23	240	2	Near death
	Stomach	24	225	3	Moderate shock
		25	210	4	4'
		26	175	4.5	3'
	Spleen	27	205	4.5	Violent shock
	Brain	28	190	4	2'
	Lung	29	210	4	2'
	Kidney	30	205	4	4'
		31	175	1	Nil
		32	180	3	Light shock
	Liver	33	220	4.5	Moderate shock
		34	210	4.5	1' 30"
		35	190	2	Moderate shock
Dog	Muscle	36	200	2	Violent shock
		37	235	3	Near death
		38	250	4	4'
	Brain	39	205	4	5'
		40	175	2	Near death
Guinea Pig	Stomach	41	160	3	Violent shock
		42	165	4.5	1' 30" Clot in heart
		43	175	3	Near death
	Intestine	44	215	4	Near death
		45	185	4.5	2' Clot in heart
		46	200	4.5	Near death
	Liver	47	170	4.5	1' 30"
	Kidney	48	185	2	Moderate shock
		49	200	4 (5 gm.)	Nil
		50	205	4.5	30'
	Lung	51	200	4 (7 gm.)	1' 30"
	Heart	52	250	4	2'
		53	215	2	Light shock
		54	185	3	Near death
	Muscle	55	185	4	Violent shock
		56	180	4.5	Near death
		57	180	4.5	2'
	Brain	58	155	2	Moderate shock

TABLE I—CONT'D
SHOWING THE DISTRIBUTION AND RELATIVE TOXICITY OF A POISONOUS EXTRACTABLE
SUBSTANCE IN TISSUE

SOURCE		GUINEA PIG		C.C.	RESULT
ANIMAL	TISSUE	NO.	WT.	INJECTED*	
Sheep	Muscle	59	175	2	2'
		60	175	1.5	Moderate shock
Vcal	Muscle	61	180	2	Near death
		62	200	2.5	3' 30"
	Liver	63	210	4	4'
		64	210	3	3'
		65	170	2	Light shock
Ox	Muscle	66	200	2	Violent shock
		67	180	2.5	3'
		68	170	2	4'
	Liver	69	160	2	Violent shock
		70	175	2.5	Near death
		71	250	3	Violent shock
		72	230	4	2' 30"
Hog	Muscle	73	175	2	Violent shock
		74	165	2.5	3'

*Each c.c. represents 2 gm. of tissue.

TABLE II
SHOWING THE AMOUNT OF MATERIAL EXTRACTED FROM THE TISSUES OF THE CAT AND
CONCENTRATION IN FINAL EXTRACT

ALCOHOLIC EXTRACTION				EXTRACT AS INJECTED		
Tissue	Wt.	Wt. of residue	% of tissue	Wt. of residue	% of tissue	mg. per c.c.
Stomach	69	0.934 gm.	1.55	0.283 gm.	0.47	9.44
Intestine	195	2.621	1.34	1.772	0.91	18.17
Liver	150	4.85	3.23	2.315	1.54	30.80
Kidney	62	0.934	1.51	0.805	1.30	25.90
Spleen	23	0.372	1.61	0.181	0.79	15.75
Lung	52	0.765	1.47	0.300	0.58	11.53
Heart	25	0.381	1.52	0.163	0.65	13.00
Muscle	350	8.275	2.36	6.406	1.83	36.60
Brain	67	1.410	2.10	0.732	1.09	21.85

Reaction of the Extracts.—The reaction of the extracts was usually markedly acid to litmus. Occasionally an extract of stomach or of intestinal tissue would be neutral to litmus. No ready explanation of these exceptions was apparent. The color of the extracts made it necessary to use litmus in titrating their acidity. The method of titration was to add in succession small amounts of standard alkali. Between each addition of alkali pieces of both red and blue litmus paper were applied to the solution. These were arranged on a clean glass plate, each pair being placed opposite each other and in exact order of use. The addition of alkali was continued until the reaction was clearly alkaline by both pieces of litmus. The litmus paper series passed from both red at one end to both blue at the opposite end of the series. When the strips of paper were air dry they were inspected and the neutral point was determined

by selecting from about the middle of the series that pair of papers that had the same tint. The total alkali added up to this point was therefore a measure of their original acidity. In Table III the acidity is expressed in c.c. of normal alkali required to exactly neutralize 100 c.c. of the extract. In the actual titration a more dilute alkali was employed, and the results were then calculated to the basis mentioned.

TABLE III
ACIDITY OF CAT TISSUE EXTRACT

TISSUE	C.C. NORMAL ALKALI REQUIRED TO NEUTRALIZE 100 C.C.
Intestine A	2.88
Intestine B	1.88
Liver A	2.71
Liver B	2.40
Liver C	1.81
Lung A	0.34
Lung B	1.77
Stomach A	0.67
Stomach B	1.60
Brain A	0.40
Brain B	2.43
Kidney A	2.50
Kidney B	2.73
Kidney C	2.00

The letters indicate that an extract from a different animal was being tested, although from the same tissue. Guinea pigs are susceptible to injections of acid solutions. It was thought that the acidity of the extracts might be the ruling factor in their toxicity. But no lack of toxicity was found in those extracts of stomach and intestine that were found to be neutral. It was thought best to test the matter out by neutralizing an extract and then noting its toxicity. An injection of the same extract without neutralization served as the control. Some of the extracts used were the same as those reported in the preceding table and the same letters are used to designate them. Since the dilution in no case is above three per cent it is ignored in the volume of the neutralized extract that was injected.

TABLE IV
TOXICITY OF NEUTRALIZED CAT TISSUE EXTRACTS

TISSUE	TOXICITY			
	WHEN ACID		WHEN NEUTRAL TO LITMUS	
	C.C. INJECTED	RESULT	C.C. INJECTED	RESULT
Intestine A	4	2'	4	2'
Intestine B	4	2'	4	3'
Liver A	4	1'	4	Near death
Liver B	4	Violent shock	4	Light shock
Liver C	4	1'	4	Moderate shock
Stomach B	3	4'	4	Light shock
Brain B	4	Violent shock	4	Violent shock
Kidney B	4	12'	4	Near death
Kidney C	4	3'	4	Moderate shock
Intestine	4	2' (clotted blood)	4	2' (clotted blood)
Stomach	4	2' 30"	4	5'

Apparently the acidity is not the cause of the toxicity. A careful consideration of the data in Table IV shows that the attempt to modify toxicity by adjustment of reaction failed both ways. That is, the toxicity was not appreciably lowered or raised from what it was originally.

Effect of Fatigue.—A more critical survey of the data in Table I showed that muscle tissue produced an extract that seemed to more consistently hold a high toxicity than any other one tissue. This fact caused us to consider means whereby it might be possible to radically modify the muscle tissue of an animal in its physico-chemical properties. It was proposed to extract, not only the muscle, but all other tissues of the animal and observe whether any marked change had taken place in the extract of such tissues as compared to the tissues of a normal animal. Extreme fatigue was the first to be considered. A large strong cat was selected and its urine was examined for sugar. It was found to be negative. The animal was then placed in a situation that involved a moderate but continuous muscular effort. At the end of four hours the test for sugar in the urine was strongly positive. For the next few hours the sugar remained practically constant in the urine. At the conclusion of the twenty-second hour of the exertion the urine was again negative for sugar. The presence of the sugar was used as a criterion of unusual exertion. The cat was exercised for a total of ninety hours, and then exsanguinated. The tissues were prepared and extracted as usual. Table V summarizes the injection tests of the resulting extracts.

TABLE V
TOXICITY OF EXTRACTS FROM TISSUES OF A STRONGLY FATIGUED CAT

TISSUE	GUINEA PIG		C.C. INJECTED	RESULT
	NO.	WT.		
Intestine	1	195 gm.	4	1'
	2	190	2	Moderate shock
Spleen	3	160	4	Light shock
Lung	4	185	4	Violent shock
Heart	5	160	4	Moderate shock
Stomach	6	190	4	3'
	7	160	3	3'
Muscle	8	195	1	Nil
	9	250	2	Light shock
	10	235	3	Moderate shock
	11	250	4	Moderate shock
	12	215	6	Violent shock
Liver	13	185	3	Light shock
	14	205	4.5	Violent shock
Brain	15	220	4	7'
Kidney	16	225	4	Nil

We have not as yet attempted to set up any standard of toxicity to which an extract might be compared. Therefore anything in the nature of an exact measure of toxicity is impossible. However, a consideration of the extracts whose toxicity is shown in Table V shows that their relative toxicity is rather less than the general average. This is especially true for the extract from muscle. More will be said about the interpretation of this data later.

Effect of Inanition.—In continuation of the attempt to modify the properties of an extract by antemortem treatment of the animal it was decided to try inanition. Two large, well-fed cats were supplied only with water. At the end of one week one cat was removed and its tissues examined.

TABLE VI
TOXICITY OF EXTRACTS FROM TISSUES OF CAT THAT HAD BEEN WITHOUT FOOD FOR SEVEN DAYS

TISSUE	GUINEA PIG		C.C. INJECTED	RESULT
	NO.	WT.		
Stomach	1	190 gm.	3	3'
	2	185	1	Violent shock
	3	180	2	5'
	4	250	1	Moderate shock
Muscle	5	170	3	Near death
	6	255	4	2'
	7	180	3	Near death
	8	185	3.5	Near death
Spleen	9	165	4	Violent shock
Heart	10	280	4	Violent shock
Kidney	11	185	4.5	Violent shock
	12	270	5	Violent shock
Lung	13	280	4	4'
	14	200	3	3'
	15	185	2	2'
Brain	16	180	4	Violent shock
	17	190	5	4' (clot in heart)
Liver	18	250	5	Light shock
	19	170	6	6'
Intestine	20	190	5	2'
	21	195	3	3'
	22	170	2	3'
	23	175	1	3'
	24	170	0.5	Moderate shock
	25	200	1	Moderate shock

The second cat was continued in a state of inanition for a total of twenty-four days. In the course of removing the tissues it was observed that there was still some fat left under the skin and about the viscera. Due to an error in the manipulation the final extract of these tissues was made up so that each cubic centimeter represented but one gram of tissue instead of the usual two grams. If this point be kept in mind when considering the data below it will be seen that inanition has had no apparent effect on the amount of toxic material that may be extracted from the tissues of a cat.

Second Extraction of Tissue.—The residual tissue from the cat which had been without food for one week was subjected to a second extraction with alcohol. In order to increase the possibility of this extract resulting in a toxic product the tissue residues were placed in a mortar and well ground with clean sand before being placed under the alcohol for the second time. When the alcoholic extract was evaporated the small quantity of the residue was especially noticeable. In order to compensate for the evident lessened amount of material that had been extracted by the alcohol, the volume of the physiologic salt solution that was added was reduced so that each c.c. of the final extract represented 4 g. of the original tissue.

TABLE VII
TOXICITY OF EXTRACTS FROM TISSUES OF CAT THAT HAD BEEN WITHOUT FOOD FOR
TWENTY-FOUR DAYS

TISSUE	GUINEA PIG		C.C. INJECTED	RESULT
	NO.	WT.		
Stomach	1	250 gm.	4	Light shock
	2	210	6	1 hr. 4' (clot in heart)
	3	200	5	4' (clot in heart)
Heart	4	220	5	Violent shock
	5	235	3.5	Moderate shock
Eye	6	210	4	Nil
Lung	7	245	5	Moderate shock
	8	285	7	Moderate shock
Kidney	9	235	6	Moderate shock
Spleen	10	225	6	Violent shock
Brain	11	220	5	Violent shock
	12	215	7	5'
	13	215	5	4'
Intestine	14	215	4	4'
	15	246	3	8'
	16	210	2	Violent shock
Liver	17	225	4	Light shock
	18	210	6	Violent shock
	19	240	7	Violent shock
Muscle	20	270	6	Violent shock
	21	305	8	Violent shock

TABLE VIII
TOXICITY OF EXTRACT PREPARED BY SECOND EXTRACTION OF TISSUE

TISSUE	GUINEA PIG		C.C. INJECTED	RESULT
	NO.	WT.		
Muscle	1	185 gm.	4.5	Violent shock
Lung	2	195	4.5	Light shock
Kidney	3	155	4	Violent shock
Intestine	4	200	2.5	Light shock
Stomach	5	180	2.5	Moderate shock

Extraction by Ether.—Instead of adding 2 c.c. of alcohol for each gram of moist tissue, ether was substituted in the same proportion. The further steps in the process were not altered from the general method outlined earlier. The tissue was left in the ether for one week. Upon evaporation of the ethereal extract the minute amount of residue foretold the probable outcome of the injection tests just as it had in the previous experiment dealing with a second extraction of tissue by alcohol.

It is evident that the toxic agent that is removable from tissue by alcohol is not extracted by ether under the same conditions. This fact coupled with a consideration of the ability of dilute alcohol to remove phosphatides from tissue marks the beginning of a line of experimentation that finally led to the conclusions of this work. A more detailed discussion of the line of thought involved will be reserved for later consideration. (See Table IX.)

Extraction of Dried Tissue.—For this experiment it was decided to use only rabbit muscle. The extracts from muscle tissue were about as free from

TABLE IX
TOXICITY OF EXTRACT PREPARED BY SUBSTITUTION OF ETHER FOR ALCOHOL IN
PRIMARY EXTRACTION

TISSUE	GUINEA PIG		C.C. INJECTED	RESULT
	NO.	WT.		
Intestine	1	230 gm.	5	Nil
	2	210	9	Nil
Liver	3	190	5	Nil
	4	210	9	Nil
Muscle	5	245	9	Nil
Lung	6	195	9	Nil
Heart	7	195	7 (all)	Light shock
Kidney	8	210	9	Nil
Brain	9	200	9	Nil

technical difficulties as any, and muscle tissue could be obtained in greater quantity more readily than others. Moreover, we were becoming more thoroughly convinced that a result arrived at by the use of any one tissue could be essentially duplicated with any other. Rabbit muscle was ground finely and dried on the steam bath. By alternate grinding in a mortar and drying it was reduced to a very fine powder of uniform consistency. This dried muscle was then subjected to treatment with alcohol for five days. The quantities of alcohol and of water used in making up the final extract were based upon the weight of the moist tissue, and were not modified from the usual quantities employed.

TABLE X
TOXICITY OF ALCOHOLIC EXTRACT OF DRIED RABBIT MUSCLE

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	230 gm.	3	Nil
2	270	4	Nil
3	280	6	Nil
4	275	10	2'
5	240	8	2'
6	275	7	2'
7	250	6	Nil

The striking thing about this data is the sharp break in the toxicity. It is noticed that any quantity above six cubic centimeters is fatal, whereas all doses below that amount are without effect. This is different from the graded effects that are usually shown by the injection of sublethal doses of extracts.

A portion of this extract was evaporated to dryness and the concentration of the solids determined. It was found to be 9.4 mg. per c.c. This is equivalent to but .4 of one per cent of the original tissue, and is less than any other like determination so far made. We will in this connection give another method for the concentration of solids that will soon be the subject of fuller discussion. The residue left in the crucible in which the extract was evaporated for the determination of the total solids was ignited to a white ash and its weight determined. It was found to represent .6 mg. per c.c. of the extract, or .03 of one per cent of the original tissue.

Inorganic Material in Tissue Extracts.—We have already given some typical figures concerning the total solids that are held in solution in the extracts of various tissues. While engaged on that point the possibility of the presence in the extracted material of a portion at least of the inorganic constituents of tissues was realized. It was but a simple matter to ignite the residue left upon evaporation of a measured portion of the extracts and note the ash left in the crucible. Strong heat was required to eliminate all carbon from the ash. It was found by several trials that ignition of the residue from 25-50 c.c. of various extracts left an appreciable ash in the crucible. In the course of some further work we will give more exact expression to the quantity of the ash.

Speculation as to the chemical constituency of such an ash was hardly necessary. In the first place it is evident that nothing could be present that had not been derived from the tissues. By reference to König's analytical work it was possible to note what the theoretical composition should be for all the tissues under examination. A part of the chlorides probably would be volatilized by the temperature of ignition, which at the same time would convert the principal element phosphorus to a pyrophosphate salt with the potassium, calcium, magnesium and other basic elements present. The ash was slowly and nearly totally soluble in water, the resulting solution being alkaline to litmus. The slight insoluble portion was at one time filtered from a solution of an ash and the volume brought back to exactly that of the original extract. A small dose of this solution was then injected into a guinea pig intravenously. A typical anaphylactic shock and death ensued. The ash in this instance had been derived from an extract of cat muscle. The amount of material in solution of the ash was found to be equivalent to 8.1 mg. per c.c. To state the same thing in another form, that is, in terms of tissue, since one c.c. of the ash solution was equivalent to 2 g. of the tissue, the inorganic ash was equivalent to 4.05 mg. per gram of tissue. Injection of 2.5 c.c. of this solution was fatal in two minutes. In the following tabulation the toxicity of the ash solutions derived from the tissues is given in comparison to the toxicity of the original extract. All the extracts were of uniform tissue value; each c.c. represented 2 g. of tissue. There was some variation in the concentration of the ash solutions derived from the extract residues. The tissue value of the ash solution of the cat muscle and of both the rabbit muscles was the same as the extract, 2 g. of tissue per c.c. The tissue value of the remaining four ash solutions was four grams per c.c. or just twice that of the others.

Consideration of the data in Table XI shows that the inorganic constituents of tissue extracts when reduced to an ash by strong ignition and then injected in water solution have a toxicity equal to in most cases and greater in some than the original extract. It is realized that the toxic agent of an ash solution can not be identical with that of the extract. There is one striking feature noted above that undoubtedly corroborates this point. It will be observed that autopsy shows that the lungs of all animals dying from an injection of ash solution are collapsed. This condition is directly opposed to the distended lungs found after death from the original extract. The contrast was very

TABLE XI
SHOWING TOXICITY OF ASH SOLUTIONS AND COMPARING THEIR TOXICITY WITH THAT OF
ORIGINAL EXTRACT

ANIMAL AND TISSUE	M.L.D. OF ORI- GINAL EXTRACT	ASH SOLUTION			
		GUINEA PIG		C.C. INJECTED	RESULT
		NO.	WT.		
Cat muscle	4 c.c.	1	200 gm.	2.6	Light shock
		2	180	2.3	2' lungs collapsed
Sheep muscle	2	3	150	1	4' lungs collapsed
		4	175	1	Near death
		5	155	1	Violent shock
		6	180	1.25	3' lung collapsed
Veal muscle	2.5	7	160	1.5	2' lungs collapsed
		8	210	1.5	5' lungs collapsed
		9	200	2	2' lungs collapsed
		10	185	1	Nil
		11	190	1.5	Moderate shock
Beef muscle	2	12	170	2	2' lungs collapsed
		13	165	1	1' lungs collapsed
Hog muscle	2.5	14	200	1.5	5' lungs collapsed
		15	180	1	Nil
		16	200	1.5	Violent shock
		17	180	1.5	2' lungs collapsed
Rabbit muscle A	3	18	260	3	Violent shock
		19	230	4	2' lungs collapsed
Rabbit muscle B	3	20	260	3	2' lungs collapsed
		21	215	2	2' lungs collapsed
		22	225	1.5	Violent shock

marked. The distention on the one hand seemed to be nearly always maximum, while the collapse was apparently to the minimum. No other differences were observed upon autopsy of the animals killed by the ash solution.

The toxicity of the ash solutions turned our attention to the inorganic elements of the extracts. It was beyond reason to believe that the toxic agents of the extracts and of the ash solutions were the same. However, there is another possibility that could not be so easily disposed of. The exact condition of the inorganic material in living tissue is as yet uncertain. The probabilities are that most of it is in some sort of combination with the organic or nitrogenous portion of the tissues. Tests for acid radicals in the extracts were weakly indicative of their presence. The nature of the extracts made the interpretation of such tests precarious. So it must remain as doubtful whether the inorganic material of the extracts, acting as such, had any part in the original toxicity. This possibility is the subject of an experiment that will be reported later. However, it is not outside the realm of possibilities that the inorganic constituents in whatever combination they might have been, in the extracts, were the cause of their toxicity. This latter idea was therefore tested out experimentally.

Comparison of Alcoholic and Water Extracts.—It will be recalled that this study was evolved out of an attempt to recover Vaughan's poisonous protein cleavage product from the tissues of a cat that had been dosed enterally with the poison. Alcohol was employed as the extracting medium for two reasons. The first was the belief that the protein coagulating power of the alcohol would tend to eliminate much of the extractives of tissue. Secondly, the poison in question is more soluble in alcohol of any concentration than in

water. It was therefore expected that the differential action of the alcohol would favor its recovery from the tissue if it was present. The fact that when the absolute alcohol was added to the moist tissue it was no longer absolute was appreciated. Using the commonly accepted figures for the proportion of water in tissues and assuming an intimate intermingling of the water with the added alcohol it became about seventy-three per cent alcohol. This dilute alcohol was evidently extracting from the tissues a considerable portion of the inorganic part in whatever form it may be in the tissue. It was assumed that the inorganic material might be more soluble in water than in dilute alcohol. If this assumption should prove correct and the inorganic materials were in any manner concerned with the toxicity of the extracts, it would result in a more toxic extract. Hence it was decided to use water as the primary extracting fluid in place of the alcohol. Rabbit muscle was the tissue first subjected to such extraction. It was thought proper to make the first extraction parallel a portion of the same tissue by the alcoholic method. Then a comparison of the essential characteristics of the resulting extracts could be made. Therefore a rabbit was stripped of its muscles and they were ground as usual. The tissue was then divided into two equal parts and one was placed under alcohol, the other under water in the same proportion, 2 c.c. per gram of tissue. To the water extract formol was added to three per cent to restrain bacterial action. The final extracts are designated below as "alcohol" and "water." It is understood that the treatment of the residues left from the evaporation of the extracting liquids was identical for both and was no different from the usual method.

TABLE XII
COMPARISON OF EXTRACTS MADE FROM RABBIT MUSCLE BY ALCOHOLIC AND WATER EXTRACTIONS

	ALCOHOL				WATER			
	GUINEA PIG		C.C. IN- JECTED	RESULT	GUINEA PIG		C.C. IN- JECTED	RESULT
	NO.	WT.			NO.	WT.		
Toxicity of extracts	1	240	3	2'	1	220	3	2'
	2	255	2	Light shock	2	215	2	2'
					3	225	2	3'
					4	225	1	Light shock
Toxicity of ash solution*	1	260	3	2'	1	200	2	2'***
	2	215	2	2'	2	210	1.5	3'
	3	225	1.5	Light shock				
Concentration of solids	Total solids 33.4 mg./c.c. or 1.67% of tissue Ash 6.32 mg./c.c. Or .32% of tissue				Total solids 36.0 mg./c.c. Or 1.80% of tissue Ash 9.5 mg./c.c. Or .48% of tissue			

*Volume of ash solution equivalent to extract from which derived.

**Lungs of all animals dying from ash solution were collapsed.

Certain aspects of the above data are worthy of some consideration. It is evident that the water extraction has removed more solids from the tissue than the alcohol. The increase is 7.8 per cent. This is not a marked increase. However, the ash determination tells the real difference in the extracts. The increased quantity of inorganic material in the extract made with water is ex-

TABLE XIII

SHOWING HOW TOXICITY OF EXTRACTS CAN BE CONCENTRATED BY FREEZING

SOURCE		M.L.D. ORIGINAL EXTRACT	NO. OF FREEZINGS	GUINEA PIG		LAYER AND C.C. IN- JECTED	RESULT
ANIMAL	TISSUE			NO.	WT.		
Cat	Muscle	4 c.c.	1	1	190	2 top	Nil
				2	220	4 "	Light shock
				3	210	2 lower	2'
				4	225	1 "	2'
				5	220	0.5 "	Light shock
			5	6	220	4 top	Nil
				7	185	0.5 lower	Near death
				8	210	1 "	1' 30"
				9	265	4 top	Nil
				10	230	2 lower	5'
Cat	Intestine	5	4	11	220	1.5 "	Near death
				12	205	1 "	Near death
				13	210	4.5 top	Violent shock
				14	170	1 lower	1'
Rabbit	Muscle	3	5	15	180	1 "	4'
				16	210	4 top	Nil
				17	200	1 lower	2'
Rabbit	Liver	4.5	5	18	160	0.5 "	3'
				19	205	4 top	Nil
Rabbit	Intestine	4.5 (moderate shock only)	5	20	170	1 lower	Near death
				21	165	2.5 top	Nil
Dog	Muscle	4	1	22	155	1.5 lower	Violent shock
				23	210	4 top	7'
				24	220	1 lower	Violent shock
				25	160	2 "	2' (clot)
Guinea pig	Muscle	4	3	26	185	4.5 top	Nil
				27	210	2 lower	Near death
				28	180	2.5 "	2'
Guinea pig	Liver	4.5 (near death)	3	29	190	4.5 top	Nil
				30	185	2 lower	Violent shock
				31	180	3 "	Near death
				32	220	4 "	3'
				33	175	4 top	7'
				34	185	0.5 lower	Violent shock
Sheep	Muscle	2	3	35	185	1 "	Near death
				36	190	4 top	Nil
				37	260	1 lower	3'
Veal	Muscle	2.5	4	38	185	0.75 "	Near death
				39	250	4 top	Nil
				40	210	1 lower	3'
Hog	Muscle	2.5	4	41	155	0.5 "	Violent shock
				42	240	4.5 top	3'
				43	240	2.5 "	Nil
				44	225	1 lower	2'
Beef	Muscle	2	3	45	220	0.5 "	Near death
				46	215	3 top	Moderate shock
				47	245	1 lower	3'
				48	250	0.75 "	Moderate shock
				49	185	6 top	Violent shock
			5	50	245	8 "	3'
				51	195	1 lower	2'
				52	215	0.5 "	2'
				53	200	0.5 "	1'
				54	190	0.25 "	Near death
				55	230	0.5 "	Near death

actly 50 per cent more than that in the regular alcoholic extract. It is also observed that the water extract has a higher toxicity than the alcoholic, which is true as well of their respective ash solutions.

Effect of Freezing.—A mixture of salt and finely cracked ice was prepared. Portions of extracts in large test tubes were plunged into such a freezing mixture and allowed to remain until frozen solidly. The tubes were withdrawn and their contents slowly thawed by standing at room temperature. The first time that this was done it was observed that the color of the extract was slightly deeper in the bottom of the extract and correspondingly faded out in the upper portion. The intermediate area was a transition zone between the conditions at the opposite ends. A slight coagulum occupied a position just above the lower, more deeply colored portion. By means of a bulb pipette with a fine bore stem it was possible to withdraw some of the bottom part without disturbing the upper layers of the tube. Withdrawal of the top portion was of course readily accomplished. A comparison of the toxicities of the top and bottom portions showed an increased toxicity of the lower more deeply colored layer and a decreased toxic effect of the top less deeply colored part. A second freezing and thawing augmented the separation into layers that had been initiated by the first freezing and made a sharper line of demarcation between the bottom layer and the coagulum immediately above it. The bottom layer after the second freezing occupied about one-fourth to one-sixth of the total depth of the fluid in the tubes. Repeated freezing and thawing continued the layering process until the upper one-third to one-half of the fluid was nearly water white. The most of the color was concentrated in the bottom layer. The transition zone became lessened in extent necessarily. It was no longer a true transition zone excepting upwards. The break between the lower part and the coagulum area was sharp. This maximum effect could be obtained by five or six freezings and thawings. A larger number did not carry the effect any farther. In fact it could not be carried to a better separation on account of the diffusion that went on simultaneously with the thawing of the ice.

All the extracts in Table XIII were prepared by the use of alcohol as the primary extracting fluid. The last tissue in Table XIII is the extract of rabbit muscle prepared alongside the extraction of another portion of the same muscle by water. The data concerning this extract were given in Table XII. The freezing process was applied to the water extract, it being repeated four times. The separation into an upper watery layer and a lower colored layer was remarkably complete and sharp. Further, there was no coagulum anywhere in the tube. The injection tests gave the results as shown in Table XIV.

TABLE XIV
RESULT OF FREEZING AN EXTRACT OF RABBIT MUSCLE PREPARED BY USE OF WATER ONLY

GUINEA PIG		LAYER AND C.C. INJECTED	RESULT
NO.	WT.		
1	240	4 top	Nil
2	270	6 "	Nil
3	230	1 lower	2'
4	225	0.5 "	3'
5	200	0.25 "	2'

Portions of the top and lower layers of this extract were subjected to a determination of their solids and ash by the usual methods of evaporation and ignition. The resulting values were found to be:

	TOP LAYER	BOTTOM LAYER
Total solids	3.5 mg./c.c.	180.4 mg./c.c.
Ash	0.9 mg./c.c.	46.7 mg./c.c.

The ash resulting from the treatment of the bottom portion was taken up in water and the insoluble part filtered out. The volume was adjusted to twice that of the extract represented, so that each c.c. was equivalent to one gram of tissue. Injection of 0.5 c.c. of this ash solution was fatal to a 200 g. guinea pig in two minutes. The lungs were collapsed. It might be of some interest to give the figures showing how the solids in solution were concentrated in the lower layer for one more extract. The extract in question is the last one in Table XIII, made from rabbit muscle.

TABLE XV

SHOWING THE EFFECT OF FREEZING ON THE DISTRIBUTION OF SOLIDS IN RABBIT MUSCLE EXTRACT

	TOTAL SOLIDS	ASH
Homogeneous extract	30.67 mg./c.c.	5.64 mg./c.c.
Lower layer after two freezings	73.84 " "	14.4 " "
Lower layer after five freezings	118.6 " "	26.6 " "
Top layer after five freezings	7.35 " "	1.35 " "

The figures for the solids and ash after the five freezings would undoubtedly have been much greater had not the withdrawals of a small portion of both top and bottom layers been made for injection and determination of solids and ash after the second freezing. However, the trend of the solids to move to the bottom is well shown by the figures as they stand.

Effect of Dialysis.—If there should be two toxic substances in the extracts it was thought they might possibly be separated by dialysis. An extract of rabbit muscle was prepared, using alcohol as the extracting liquid. The final extract was made up with distilled water. Parenthetically, we may remark that this extract demonstrates that nothing is gained by a long continuation of the primary extraction period. In this case the tissues were left under the alcohol four weeks, yet the toxicity was no greater than if left for but two days as was done in some instances. Three c.c. killed in two minutes; 2 c.c. resulting in a violent shock only.

Collodion sacs were prepared by the Novy method and mounted on cut off test tubes of appropriate size. In each case the sacs were tested for leaks before using. They were then filled with the extract to the glass line. This point furnished a convenient mark from which to note and, if desired, to measure the endosmosis. The amount of endosmosis could be accurately determined by difference between the volume of the extract placed in the dialyzer and the volume of water required to fill the sac to the liquid line at the conclusion of the

dialysis. For the first dialysis 40 c.c. of extract was used, and was continued for 24 hours at room temperature against distilled water running so that one could count the drops as they fell from the overflow. Endosmosis was 5 c.c. The increase is ignored in all volumes and figures for concentration of solids. The toxicity of the dialyzed extract was the first point to claim attention.

TABLE XVI
TOXICITY OF DIALYZED EXTRACT OF RABBIT MUSCLE

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	195	3	Nil
2	190	5	Moderate shock
3	200	8	3'
4	190	6	Light shock

As the M.L.D. of the undialyzed extract was 3 c.c. it is evident that the toxicity has materially decreased. In looking for an explanation of this, the fate of the solids in solution was determined. Measured portions of the dialyzed and undialyzed extracts were subjected to the usual process of evaporation and ignition.

TABLE XVII
EFFECT OF DIALYSIS ON CONCENTRATION OF SOLIDS

	TOTAL SOLIDS	ASH
Undialyzed extract	30.67 mg./c.c.	5.64 mg./c.c.
Dialyzed extract	5.8 " "	0.4 " "

The results of this experiment pointed clearly to a close relationship between the toxicity of an extract and the solids held in solution. The fact that the material in solution did in large part dialyze out so readily settled its status in the extract. It was at one time thought that the solids might be in a colloidal solution, or possibly in a larger suspension or emulsion. Inability to remove any appreciable amount of material by high speed centrifugation, accompanied by an unaltered toxicity, made the notion of a suspension or emulsion no longer tenable. The only conclusion left, that of a true solution, is made much more plausible by the results of dialysis.

However, we were no nearer the truth concerning the possibility of two substances in the extract than before. It was decided to continue the investigation of this point by varying the conditions of the dialysis or the permeability of the sacs. The following protocols give the details involved:

(a) Rabbit muscle was extracted with alcohol and made up with distilled water. The toxicity of the original homogeneous extract was:

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	225	3	1' 30"
2	250	2	Near death

A very thin sac was prepared and a portion dialyzed for seven hours. The endosmosis was very marked and required a factor of .812 to be applied to the final volume in order for it to become comparable to the original extract. This correction is made in the volumes in the injection tests that are here reported for the dialyzed extract.

TOXICITY OF EXTRACT DIALYZED SEVEN HOURS

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	220	2.4	Nil
2	245	3.2	Nil
3	225	4.9	Light shock
4	265	5.7	Moderate shock

The values for the solids and ash in the dialyzed and undialyzed extracts were found to be:

	TOTAL SOLIDS	ASH
Undialyzed extract	33.0 mg./c.c.	6.34 mg./c.c.
Dialyzed extract (7 hrs.)	9.3 " "	1.04 " "

Another portion of this extract was dialyzed for twenty-four hours in an ordinary sac. The endosmosis was slight and was ignored. An injection of six and eight cubic centimeters into two guinea pigs of 200 g. was devoid of toxic effect. No determination of solids was made on this latter portion.

(b) Another extract of rabbit muscle was prepared by alcoholic extraction. Three c.c. killed a guinea pig in two minutes. A dialyzing sac was made and mounted and then permitted to become air dry by standing at room temperature. The effect on the sac is to markedly lessen its permeability. The dried sac was then filled and placed in a bath at 45° C. for two hours. No endosmosis had taken place in this time. Three c.c. were withdrawn and upon injection killed the animal in two minutes. The dialysis was discontinued, as it was evident that the sac was so impermeable that no results could be expected.

(c) Another sac of moderate thickness was prepared, and when filled with the extract was placed in a bath that was adjusted to a temperature of 50°-55° C. The dialysis was continued for two hours. The endosmosis was quite marked, amounting to 20 per cent of the original volume. This is ignored in the injection tests and figures for solids and ash determinations.

TOXICITY OF EXTRACT DIALYZED TWO HOURS AT 50-55° C.

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	205	3	Nil
2	280	6	5'
3	275	5	Nil
4	290	6	Nil

The determination of solids and ash gave the following results:

	TOTAL SOLIDS	ASH
Undialyzed extract	33.4 mg./c.c.	6.32 mg./c.c.
Dialyzed extract	4.95 " "	Nil

It is to be observed that the figures for both the total solids and the ash in the undialyzed extracts reported above in "a" and "c" are quite close together. These extracts were made from the tissue of different rabbits on separate occasions. Such agreement as this is at least indicative of a possibility of the preparation of a consistent extract by following closely the same technic. As far as these dialysis experiments are concerned, it is apparently impossible to make any separation of the constituents of an extract by differential dialysis.

Effect of Boiling Extracts.—On three occasions a muscle extract was boiled to observe whether there was any alteration of its toxicity. The first time an extract of cat muscle was actively boiled in a large test tube over an open flame for five minutes. A fine coagulum was formed in the extract. The boiled extract was divided into two portions and one was filtered. Then injections were made of both the filtered and the unfiltered boiled extract. As a control, injection of unboiled extract was made. No differences could be detected in the effects of the three portions.

The next extract that was boiled was also from cat muscle. It had a slight opacity. Boiling one minute caused this to disappear. No coagulum was formed in this extract. Cooling under the tap did not restore the opaque appearance of the extract. No change in toxicity could be noted. Finally an extract of rabbit muscle was boiled five minutes. A coagulum similar to that produced in the extract of cat muscle made its appearance. The boiled extract was injected both filtered and unfiltered. No differences could be observed between these two portions and an unboiled portion. We believe that these tests warrant the assertion that boiling has no effect on the toxicity of a muscle extract.

Filtration of Muscle Extract Through a Berkefeld.—An extract of rabbit muscle was prepared with water as the primary medium of extraction. A portion was then passed through a Berkefeld "N" filter. As far as appearances were concerned the extract came through unaltered. Table XVIII shows that the toxicity remained constant also.

TABLE XVIII
 FILTERING RABBIT MUSCLE EXTRACT THROUGH A BERKEFELD

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	170	1 filtered	Nil
2	170	1 "	Violent shock
3	160	1.5 "	3'
4	170	1.5 "	3'
5	165	1 unfiltered	Violent shock
6	170	1.5 "	3"

The high toxicity of this extract may be noted. It was prepared by the use of water as the primary medium of extraction. We have already cited the experimental data showing the essential differences between an alcoholic and a watery extraction. This extract substantiates our contentions then made concerning extracts made with water.

Phosphates and Neutrality.—Inasmuch as we wished to do some work on the inorganic ash it was decided to prepare a reasonably large quantity of it. Therefore 966 g. of rabbit muscle was extracted with water and the final extract made up with distilled water to 483 c.c. No test of the toxicity was made, but it was all evaporated and the residue ignited. The resulting ash was taken up in the same volume of water and the insoluble portion filtered out. This

ash solution was evaporated to dryness and the residue was scraped together, carefully ground in a mortar, dried and weighed. The product thus obtained represented the water soluble portion of the inorganic ash, that had been extracted from the rabbit muscle (966 gms.) by water. It was found to weigh 4.54 g. This was equivalent to 4.7 mg. of ash from each gram of the moist tissue. The concentration, therefore, in the water solution was 9.4 mg. per c.c. Or the ash was 0.47 of one per cent of the tissue. A one per cent solution of this ash was made up. This solution was nearly of the same concentration as the original solution of the ash when the whole quantity was in 483 c.c. of water. Calculation shows that solution to have been .94 of one per cent. The one per cent solution was perfectly clear and water white. It was alkaline to litmus and phenolphthalein. One c.c. injected intravenously into a guinea pig was fatal in four minutes. Twenty-five c.c. of this one per cent solution required .25 c.c. of normal succinic acid to discharge the pink color of the one drop of phenolphthalein indicator that had been added. The toxicity of the neutralized solution was unaltered. Portions of the solution were also carefully neutralized with acetic, hydrochloric, and sulphuric acids. In each instance the toxicity remained unchanged.

The application of qualitative chemical tests to the solution of the ash showed the presence of chloride and sulphate radicals in rather minute amounts. By the use of differential tests the pyrophosphate radical was shown to be present in considerable quantity. An attempt was made to precipitate out the pyrophosphate radical by careful titration with silver nitrate. Two methods of determining the end point were employed. One, and the most successful, was to let the precipitate settle and then carefully add another drop of the reagent, noting whether a precipitate formed at the point where it was added. The other method used was that of potassium chromate as an indicator of free silver ions. A drop of the ash solution that was being precipitated by the silver nitrate was added to a drop of chromate indicator and the presence or absence of silver chromate noted. When the end point was reached the solution was allowed to stand for some time for the precipitate to settle out. Then a few c.c. of the supernatant liquid was withdrawn by a bulb pipette. Injection of this into guinea pigs showed that the toxicity had been reduced about one-half. That is, where the M. L. D. of the original ash solution was one c.c., after precipitation 2 c.c. could be injected and one-half of the pigs lived. All animals receiving injection of the precipitated ash solution showed a shock of some severity. Not a great deal of reliance can be placed on this experiment. It was shown by appropriate controls that the slightest excess of silver nitrate would produce death. In attempting to titrate to an end point in which the presence of the added ion in an unbound condition was used as the indicator it was practically certain that sufficient of these silver ions were present to upset any rigid interpretations of the injection of such a treated ash solution. However, it is significant at least that when the pyrophosphate was precipitated the toxicity showed some decrease.

The same experiment was tried with barium chloride as a precipitant, but with less success.

The phosphorous in the ash solution was determined by a standard volumetric method. It is not necessary to give the details of the procedure, since they were the same as would be employed by anyone for the same purpose and are in any standard textbook of volumetric analysis. It was determined that the ash contained phosphorus, calculated as phosphorus pentoxide to the extent of 34.83 per cent. Extending the significance of this value, it means that injection of 3.48 mg. phosphorus pentoxide, when in the form of pyrophosphate radical, is fatal to a guinea pig.

Toxicity of Pure Phosphate Salts.—It was decided to test the very obvious indication just brought out concerning the toxicity of pyrophosphate radical by the use of pure salts. For these tests Kahlbaum's salts were employed. However, due to the uncertainty of hydration in phosphate salts, uniform solutions were employed, and analytical results relied upon for information concerning the exact quantity of material in solution. The data will be given in form of protocols.

SODIUM ORTHO-PHOSPHATE

A one per cent solution was prepared. It was alkaline to litmus and phenolphthalein. Injection of this solution gave the following results:

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	170	1	Nil
2	190	4.5	Nil

Twenty-five c.c. of the solution required .7 c.c. of normal succinic acid to neutralize to phenolphthalein. Injection of 4.5 c.c. of the neutralized solution was still entirely without effect. Volumetric analysis showed phosphorus calculated as phosphorus pentoxide to be 24.9 per cent. Therefore, 11.2 mg. of phosphorus pentoxide, when in the form of orthophosphate radical is without any effect upon injection into guinea pigs.

SODIUM METAPHOSPHATE

A one per cent solution was made up. It was acid to litmus and phenolphthalein. The injection tests resulted as follows:

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	180	1	3'
2	170	0.5	Moderate shock
3	185	0.75	3'
4	165	0.75	2' 30"
5	185	0.5	Light shock

Twenty-five c.c. of this solution required .33 c.c. of normal sodium hydroxide solution to neutralize to phenolphthalein. The injection of the neutralized solution gave the following results:

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	170	1	2
2	150	0.75	Violent shock
3	150	0.75	Violent shock; dead in 12 hours
4	150	1	Violent shock; dead in 12 hours
5	205	1	Violent shock; slow recovery
6	215	1.25	2'

Volumetric analysis showed the presence of 57.48 per cent of phosphorus as phosphorus pentoxide. Hence for unneutralized solutions of sodium metaphosphate, 4.32 mg. phosphorus as the metaphosphate radical is fatal for the guinea pig. When the solution is made neutral to phenolphthalein the amount is increased to 7.18 mg.

SODIUM PYROPHOSPHATE

In order to make more certain that the salt was all in the form of pyrophosphate it was strongly ignited before using. A one per cent solution was employed as before. The solution was alkaline to litmus and phenolphthalein. Injection of this solution gave the following results:

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	185	1	2'
2	200	0.5	2'
3	180	0.25	Light shock
4	235	0.5	Violent shock
5	225	0.5	10'

The one per cent solution was diluted with distilled water volume for volume, thus making it a .5 per cent solution. Injection of the diluted solution resulted as below:

GUINEA PIG		C.C. INJECTED	RESULT
NO.	WT.		
1	205	1	3'
2	195	0.5	Nil
3	215	1	3'

Twenty-five c.c. of the one per cent solution required .5 c.c. of normal succinic acid for neutralization to phenolphthalein. Injection of the neutralized solution showed that the toxicity was the same as before. Volumetric analysis gave 52.85 as the value for phosphorus calculated as phosphorus pentoxide. The theoretic amount for a pyrophosphate salt is 52.97 per cent. Therefore, 2.64 mg. of phosphorus pentoxide in the form of a pure pyrophosphate salt is fatal for a guinea pig. Neutralization of the solution does not alter the toxicity.

The lungs of all animals dying from an injection of any of the sodium salts or the salts recovered from the extract were collapsed, and pink in color. The blood was in all cases fluid, the heart in one timed instance continuing to beat for one hour and forty-five minutes. The parallel between the toxicity of the pyrophosphate salt and the salts from the extract is the outstanding feature of this experiment. It seems sufficiently established by this test that the toxicity of the ash from the extracts is due to their content of potassium pyrophosphate.

Toxicity of Rabbit Muscle Extract for Rabbits.—An extract of rabbit

muscle with an M.L.D. of 1.5 c.c. for 200 g. guinea pigs was prepared by the use of water only. Some half grown and baby rabbits being available it was decided to make a few injections of this extract and note its toxicity for the homologous animal.

TABLE XIX
TOXICITY OF RABBIT MUSCLE EXTRACT FOR THE HOMOLOGOUS ANIMAL

RABBIT		C.C. INJECTED	RESULT
NO.	WT.		
1	280	2.1	Nil
2	275	4.2	1' 30"
3	1085	8.2	Nil
4	1290	20.0	2'

In the tests in Table XIX rabbits number one and three received an injection that was fatal for guinea pig per kilogram of weight. Rabbits number two and four received twice the fatal quantity for guinea pigs per kilogram of weight.

Experiments with Serum.—On several occasions during the progress of the experimental part of this work an attempt was made to modify the toxicity of extracts, by the addition of rabbit serum and guinea pig serum. As far as the use of the serum of normal animals is concerned we are prepared to state that it is entirely without effect.

By repeated small injections an attempt was made to immunize rabbits against rabbit muscle extract. The injections were one-tenth the fatal quantity per kilogram of guinea pig, calculated on the weight of the rabbits. The injections were made every other day in the ear veins. On the third injection one of the rabbits gave the typical cry of an anaphylactic rabbit and was dead in two minutes. A second rabbit died on the fifth injection, notwithstanding a reduction of the dose to one-half the amount used in the beginning. The third rabbit successfully received six injections, but lost one hundred grams in weight. The animal was kept one week after the last injection and the serum secured by the usual process of bleeding in a pipette, rod defibrination, followed by immediate whirling out of the cells. This serum, possibly containing antibodies against the extract, was then added to one M.L.D. of the same extract that had been injected into the animal, and the resulting mixture tested on guinea pigs. The results of the injections are given in Table XX. Another series of mixtures of the serum and extract was prepared and then incubated at 37° C. for one hour. Following the incubation, the tubes stood at room temperature for fifteen hours and then the injections were made. The data is in Table XXI.

It is seen that the effect of the serum is nothing more than that of dilution, for the same slight evidences of weakening of toxicity can be produced by the use of physiologic salt solution. The attempt to immunize the rabbits also shows that the effect of repeated small injections apparently becomes cumulative. However one may try to explain the deaths of the rabbits, the fact remains that they were killed by a total quantity of extract that amounts to .3

TABLE XX

TOXICITY OF RABBIT MUSCLE EXTRACT WHEN MIXED WITH SERUM OF A RABBIT THAT HAS BEEN SUPPOSEDLY IMMUNIZED

GUINEA PIG		C.C. EXTRACT	C.C. SERUM	C.C. PHYSIOLOGICAL SALT	RESULT
NO.	WT.				
1	190	1.5	0.5	0	5'
2	185	1.5	0.5	0	3'
3	225	1.5	1.0	0	Violent shock
4	205	1.5	1.0	0	6'
5	215	1.5	1.5	0	Violent shock
6	210	1.5	1.5	0	10'
*7	250	1.5	0	1.5	Violent shock
*8	200	1.5	0	1.5	2'
*9	215	1.5	0	0	3'

*Controls.

TABLE XXI

TOXICITY OF RABBIT MUSCLE EXTRACT WHEN INCUBATED WITH SERUM OF SUPPOSEDLY IMMUNIZED RABBIT FOR ONE HOUR; ROOM TEMPERATURE FIFTEEN HOURS

GUINEA PIG		C.C. EXTRACT	C.C. SERUM	C.C. PHYSIOLOGICAL SALT	RESULT
NO.	WT.				
1	250	1.5	0.5	0	Violent shock
2	200	1.5	0.5	0	3'
3	205	1.5	1.0	0	Violent shock
4	210	1.5	1.0	0	5'
5	200	1.5	1.5	0	3'
6	200	1.5	1.5	0	Violent shock
7	200	1.5	2.0	0	Violent shock
8	180	1.5	2.0	0	Violent shock
*9	200	1.5	0	1.5	6'
*10	245	1.5	0	1.5	Violent shock
*11	250	1.5	0	2.0	Near death
*12	220	1.5	0	2.0	Violent shock
*13	200	1.5	0	0	3'
*14	210	1.5	0	0	5'

*Controls.

in one case and .5 in the other of the quantity that would have been required to have killed them in one single injection on the assumption that rabbits are as susceptible as guinea pigs.

DISCUSSION

The first or historical part of this paper requires but little further consideration. The reading of it has no doubt left the impression that there is yet considerable confusion in the field of organ extracts. Direct contradiction prevails on some points and agreement on certain others; but by far the larger number of questions involved are still in the balance. The one question that all have striven to answer, directly or indirectly, is the identity of the toxic agent in organ extracts.

To reflect for just a moment on the lack of agreement among the workers in this field. By a more careful reading of the details of their work one can see a plausible reason why such variant conclusions have been reached. When

one takes into consideration the diversity of workers, and hence of technic, the wide range of materials that have been examined, and the variety of animals that have been used as recipients upon which to test toxicity, it is seen at once that sharp conflicts of opinion are inevitable. Probably the variation in the technic of extraction, together with the use of different animals as recipients are the most potent factors in this matter. The latter point we believe to have especial significance. If the guinea pig may be said to be too sensitive in its response to injections, the converse is probably true of all other laboratory animals, and especially of the rabbit, which has been used so extensively in organ extract work. To illustrate, if one injects a series of half a dozen guinea pigs with graduated doses of some toxic substance, within limits, all give some definite response to the injections. The gap between no response and an easily observable effect will be relatively small in terms of dosage. This is not so for the rabbit. They receive with equanimity relatively large doses of toxic material, even bordering on the fatal quantity. Thus it is seen how two independent operators could easily arrive at opposite conclusions. One by injecting doses into rabbits that were just under the M.L.D. would get only negative results; the other by use of a quantity but slightly larger, yet over the fatal amount, makes a positive observation. Both are correct in their observations, yet the published work seems absolutely contradictory at first reading. Our personal experience with rabbits strongly corroborates this possible explanation of some of the confusion in the literature of organ extracts.

The statement made earlier concerning the circumstances under which this study was initiated may be recalled. The extraction process that was then employed is admittedly a crude procedure. However, it resulted in a toxic extract from the tissues of a normal cat. It was the substance that was in that extract that we wished to know more about, therefore the technic by which it was obtained has been quite closely followed throughout this work. It can be readily understood, that in extracting such a complex material as tissue, that any radical change in method would result in a corresponding change in the extract. The attempt to extract the tissue with ether in place of the alcohol well illustrates the point. Or, the previous drying of the tissue, followed by alcoholic extraction, which produced an extract of such negligible toxicity. The lessened toxicity of the extract from the tissues of the fatigued cat is not so readily explained. Various factors were influencing the cells of that animal during the period of fatigue, whereby they could have been markedly modified. The exertion, lack of food, worry, and the necessity of metabolism of something as a source of energy, is a combination that has many possibilities of altering an organism. The experiment on inanition alone rules out that factor, as the cat subjected to that test showed no effects of the treatment in the resulting extract of its tissues. Without going into the theoretical aspects of the matter, it may be said that authorities are agreed that two changes would ensue in the fatigued animal. One would be a change of cell contents; a transfer of energy producing material from certain cells to others more vitally concerned with the maintenance of life, such as the heart and nervous tissues. From the

viewpoint of our purposes, the change that probably took place in the cell membranes was the more potent. This assumes of course that the toxic agent of organ extracts is a constituent of cells, and must of necessity be removed through the membranes in extraction. Even if there was no change whatever in the cell contents, the decreased permeability of the membranes is very likely. This is shown by the failure of the extraction process to remove anywhere near the usual amount of toxic substance from the tissues, judging by the results of the injections.

Up to the time when water was employed as the extracting medium the extraction process had not been aimed at any particular substance. It has already been explained how our efforts of extraction became more concerned with the inorganic constituents of the tissues. The primary extraction with water resulted in an enormous increase in the bulk of the extractives removed from the tissue. However, by carrying the evaporation of the water to absolute dryness, much of this material was so changed that it was not taken up by the next treatment with the water. The data shows the actual increase of total solids to only be about eight per cent over that contained in the extract prepared by the use of alcohol as the primary extracting liquid. The increase in the inorganic matter as determined by the ignition was approximately fifty per cent. The size of dose required to kill was reduced in the case of rabbit muscle to about one-half what it had been when alcohol was used for the extraction. It would seem that the connection between the inorganic part of the extracts, in whatever form they may be, and the toxicity is close enough to warrant the belief that the toxicity is caused by this part of the material in solution. The exact form in which the inorganic material was disposed is an unsettled question. Undoubtedly some small part of it is free inorganic salts. It was found impossible to separate the inorganic from the organic substance except by ignition. Dialysis might have accomplished this result but failed to do so. This points very strongly to the probability that the inorganic elements were in combination with something else. The matter can be considered from the standpoint of the principal radical present most advantageously. As has been shown the acid radical most abundant in the ash is the phosphorus. When one considers the possible forms of combination of phosphorus in the animal body, the so-called "phosphatides," in relation to the method of extracting the tissues, it will be seen that lecithin is the most probable combination that fits the circumstances of the extraction. Further the widespread distribution of lecithin and the extractable poison would support such a view. However, lecithin is said not to dialyze. Even its solution in water is somewhat dubious. These statements are made for reasonably pure lecithin, and it is certain that we are not working with a pure specimen of anything, whatever its identity may be. All laws of solubility and behavior of substances in solution are based on data from the behavior of the pure substance. When two or more substances, even when chemically pure, are present in a solution, the result of their status is not conformable to any fixed rule for either alone. The condition when such a complex as organ extracts are placed in water would not be the most simple. This

it is believed explains the situation with respect to our tissue extracts. Disregarding entirely what the toxic agent may be, the complexity of the material is sufficient to rule out any rigid statement concerning solubility, dialysis, or other phenomena of any one substance that may be present. One fact can not be escaped. That is that in the most toxic extracts, analysis showed over one-half of the inorganic constituents of the tissue to be present, and the toxicity rose as the ash value of the extract was increased.

Early in this work we had concluded that the toxic agent of tissue extracts was some stable substance. Anything in the nature of a ferment was excluded by the method of preparation of the extract. In the first place alcohol was used to extract the tissue; ferments are not able to withstand the concentration to which they would have been subjected. Further, some rabbit livers had been saved from time to time as the animals had been used for other purposes. They were preserved by dropping into a large bottle containing absolute alcohol at the start. The most recent of these livers had been in the alcohol for about one year. They were ground and the required amount of water added to the alcohol in which they had been kept to make the volume up to the usual 2 c.c. per gram of tissue. The final extract proved to be the most toxic for liver ever prepared; 1.5 c.c. proving fatal. Secondly, the alcoholic or water extraction liquid was heated during its evaporation, which was carried to relative dryness when alcohol was used and to positive dryness in the case of water, to a higher temperature than ferments can withstand. Then, the extracts themselves were stable on boiling. The extracts showed no deterioration on standing, either at room temperature or in the ice box. Extracts thus kept for three months showed no decrease in toxicity. Whenever an extract was kept for any length of time, it was preserved from bacterial activity by a few drops of trikresol.

The work upon the toxicity of the pure phosphate salts was for the purpose of confirming a well grounded notion as to the toxic agent in the inorganic salts derived from the extract by ignition. Gamgee, Priestly, and Larmuth³⁶ had noted the differences in the effects of the three forms of phosphoric acid as early as 1877, and had made a special study of the influence of the pyrophosphate salt on heart and circulation. They used rabbits and frogs, and made their injections subcutaneously and intravenously. Gardner and Symes³⁷ repeated their work in 1910, and confirmed the same. They ascribed the effects of the phosphoric salts to their alkalinity. Starkenstein³⁴ made a more minute investigation of the effects of the various acid radicals, that it is possible to obtain with the phosphoric acids, due to their polyvalency, and was also led to believe that the alkalinity of the pyrophosphate salts was responsible for their marked activity. Crawford³⁸ after investigating the ill results of feeding cottonseed meal concluded that the pyrophosphates produced by the method of manufacture were the cause of the deleterious effects. Thus we see that there is ample evidence that the phosphate salts, and especially the pyrophosphates are very toxic substances when introduced into the body by any channel. König³⁹ states that phosphoric anhydride constitutes from 36 to 48 per cent of the mineral part of

various forms of flesh. The salt that we prepared and subjected to analysis contained 34.8 per cent of phosphoric anhydride. It was necessarily in the pyrophosphate form by reason of the method of its preparation, and was undoubtedly the cause of death in animals injected with a water solution of the salts. The collapsed condition of the lungs of animals dying from injections of either pure phosphate salts or of the salts from the tissue extracts, was a very constant feature. If the statement is acceptable that an anaphylactized animal dies because air can not get out of the lungs, because of the constricted bronchioles, then it would be logical to believe that these animals died because air could not get into the lungs. The dyspnea was very marked, indicating a condition of air hunger exactly analogous to that of animals injected with organ extracts or anaphylatoxin.

The method of preparing the extracts would result in a low protein content. In fact, no satisfactory test for protein was ever made on an extract. The biuret test was negative. In view of the small amount of protein, if any, that was in the extracts, the attempt to immunize rabbits was inevitably doomed to not succeed. The toxic agent of the extracts we believe to have shown to be a chemical entity, and to act as such in the animal body. However, its apparent cumulative effects on repeated injection might be interpreted as a state of sensitization.

SUMMARY AND CONCLUSIONS

The tissues of seven species of animals were extracted with alcohol and found to yield a toxic extract. We believe that the tissue of all animals if similarly extracted would likewise produce a toxic extract.

It was found that the extract of the tissues of the cat, which may be considered representative of all other tissues, contained solids in solution varying from .47 to 1.83 per cent of the original tissue.

The extracts were acid in reaction with the occasional exception of the extract of stomach and intestine. Adjustment of the reaction to neutrality did not alter the toxicity of the extracts.

There was some evidence that toxicity of the extracts could be modified by previous treatment of the animals, especially by fatigue. Inanition was without effect.

Ether does not extract the toxic substance from tissue.

Drying and fine division of rabbit muscle followed by alcoholic extraction gave an extract of negligible toxicity. In view of statements that are to follow, we do not believe that the toxic agent was destroyed by the drying, but rather that it was rendered insoluble in absolute alcohol.

Ignition to a white ash of the solids in extracts prepared by the use of alcohol, shows an inorganic residue of approximately .4 per cent of the tissue. Subsequent solution of this ash to the volume of the extract from which it was obtained, followed by injection, shows it to be always equally toxic, and in some cases more so, than the extract from which it was derived.

It was found that water could be substituted for alcohol as the primary extracting medium, and that a more toxic extract resulted. Further investiga-

tion of this extract showed that the total solids had been increased by the use of water about 8 per cent, and the inorganic residue by 50 per cent.

The increase in toxicity of the water extracts, as measured in terms of reduction of size of dose required to kill, was approximately parallel to the increase of the inorganic material in the extracts. We, therefore, believe that the inorganic constituents of the extracts, in whatever form they may be, are responsible for the toxicity of the extracts.

That the inorganic substances were in some sort of combination is indicated by the failure of dialysis to make any separation of organic and inorganic materials. Whatever is in the extracts dialyzed freely.

By repeated freezing and thawing the toxic portion of the extracts could be concentrated to a very small volume in the lower part of the container in which the extracts were frozen. Five or six freezings gave the maximum effect. The increase in toxicity of the lower concentrated portion was roughly equivalent to the increase of solids in solution. The lack of toxicity in the uppermost portion of such a frozen extract was almost absolute.

Boiling does not affect the toxicity of muscle extracts.

The toxic agent of muscle extracts is not removed by passage through a Berkefeld N filter.

Pyrophosphate salts are highly toxic for guinea pigs upon intravenous injection. By comparative tests between pure phosphate salts and the inorganic salts derived from the extract by ignition, it was shown that the toxicity of the ash solutions was caused by their content of pyrophosphates.

The toxicity of rabbit muscle extract could not be modified by treatment with serum, either with or without incubation. This statement applies to both serum from a normal animal and serum from an animal that had been repeatedly injected with the extract, and might therefore have been immunized against the extract.

Rabbit muscle extract is toxic for rabbits, but in a higher proportion per kilogram of weight than for guinea pigs.

The attempt to immunize rabbits by successive small injections of extract shows that the effects of the extract by this manner of injection become cumulative and are exaggerated beyond what would have been produced by a single injection of the total dosage up to the time when the animal succumbs. Lack of protein material in the extracts forbids the use of the term "sensitization" as ordinarily understood. Yet the whole phenomena is typical of such a state.

The general trend of the experimental part of this work indicates that the toxic agent in tissue extracts is a stable chemical entity or entities. We believe that the evidence is sufficient to warrant the statement that the inorganic materials of the extracts, *especially the phosphorus*, in whatever form they may be are the toxic agents of tissue extracts.

ACKNOWLEDGMENTS

I wish at this time to express my thanks to Dr. V. C. Vaughan for his kind suggestions and advice, and for his friendly interest in the progress of this

work; also to Dr. F. G. Novy, Director of the Hygienic Laboratory, for the many privileges of the laboratory enjoyed.

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